Published in final edited form as:
Circ Res. 2008 May 23; 102(10): 1275–1285. doi:10.1161/CIRCRESAHA.108.171728.

Heparin-binding EGF-like Growth Factor Signaling in Flow-induced Arterial Remodeling

Hua Zhang[1], **Susan W Sunnarborg**[2], **K Kirk McNaughton**[1], **Terrance G Johns**[3], **David C Lee**[4], and **James E Faber**[1]

[1]Department of Cell & Molecular Physiology, University of North Carolina at Chapel Hill, NC

[2]Department of Biochemistry & Biophysics, University of North Carolina at Chapel Hill, NC

[3]Ludwig Institute for Cancer Research, Heidelberg, Australia

[4]University of Georgia, Athens, GA

Abstract

Heparin-binding EGF-like growth factor (HB-EGF) is activated by reduced endothelial shear stress and stimulates smooth muscle cell (SMC) proliferation *in vitro*. More- over, HB-EGF is augmented at sites of intimal hyperplasia and atherosclerosis— conditions favored by low/disturbed shear stress. We thus tested whether HB-EGF contributes to low Flow-Induced NUegative hypertrophic Remodeling (FINR) of mouse carotid artery. Blood flow was surgically decreased in the left and increased in the right common carotids. After 21 days, left carotid exhibited lumen narrowing, thickening of intima-media and adventitia, and increased circumference that were inhibited by ~50% in HB-EGF$^{+/-}$ and ~90% in HB-EGF$^{-/-}$ mice. FINR was also inhibited by the EGF receptor inhibitor, AG1478. In contrast, eutrophic outward remodeling of the right carotid was unaffected in HB-EGF$^{+/-}$ and HB-EGF$^{-/-}$ mice or by AG1478. FINR-induced proliferation and leukocyte accumulation were reduced in HB-EGF$^{-/-}$. FINR was associated with increased: reactive oxygen species, expression of pro-HB-EGF and TACE (pro-HB-EGF sheddase), phosphorylation of EGFR and Erk1/2, and NF-κB activity. Apocynin and deletion of p47^{phox} inhibited FINR, while deletion of HB-EGF abolished NF-κB activation in SMCs. These findings suggest that HB-EGF signaling is required for low flow-induced hypertrophic remodeling and may participate in vascular wall disease and remodeling.

Keywords

artery; flow-mediated remodeling; HB-EGF; reactive oxygen species; NF-ķB

Introduction

Vascular remodeling is directed by signals from vascular wall and blood-borne cells in response to changes in pressure, flow (shear stress), cyclic strain and pathological processes[1]. High-flow-induced positive remodeling (eutrophic vessel enlargement) and low-Flow-Induced Negative Remodeling (FINR, hypertrophic vessel narrowing) are important during normal embryonic and postnatal growth and use-dependent hypertrophy and atrophy of tissues[2]. Flow-induced proliferation and remodeling are also important in atherosclerosis, intimal hyperplasia, restenosis and bypass graft failure[3,4]. For example, branch points that are normally exposed

Correspondence: James E. Faber, Ph.D, Department of Cell and Molecular Physiology, 6309 MBRB, University of North Carolina, Chapel Hill, NC 27599-7545. Tel: 919-966-0327. Fax: 919-966-6927. E-mail: jefaber@med.unc.edu..

Disclosures: None

to low and disturbed (oscillatory) shear stress are susceptible to lipid accumulation, SMC proliferation and atherogenesis[3-5],.

Altered shear stress on endothelial cells (EC) initiates flow-remodeling mediated by mechano-sensitive elements and downstream signals including, pro-inflammatory pathways (eg, NF-κB)[4,6,7]. In fact, chronic low shear is known to promote endothelial dysfunction in arteries, characterized by increased reactive oxygen species (ROS) generation, reduced bioavailability of nitric oxide, and increased pro-thrombotic, pro-inflammatory and pro-migratory factors[5, 8]. Areas of disturbed shear stress have reduced barrier function, increased lipoprotein uptake and increased adhesiveness for leukocytes[5] that contribute, along with ECs and SMCs, to increased local ROS generation[8].

Study of vessel restructuring is largely confined to *in vivo* models, thus many of the underlying mechanisms are not well understood. In mouse[9,10], FINR of the carotid artery can be produced by ligating all of its distal branches except the thyroid or occipital arteries, which strongly reduces blood flow and shear stress[9,10]. The low and hyper-oscillatory shear stress induces intima-media hyperplasia, wall hypertrophy and lumen loss. Korshunov and coworkers reported that Axl-mediated inhibition of apoptosis contributes to FINR[7], while Sullivan and Hoying found that FGF2 deletion had no effect[9]. Berk and coworkers showed that eNOS and nitric oxide protect against FINR, whereas caveolin-1 promotes FINR, possibly as a mechano-element[11]. In mesenteric artery, Bakker et al linked FINR and tissue transglutaminase[12].

We recently reported that α_1-adrenergic activity contributes strongly to FINR[13], and, in other studies, that the trophic action of α_1-adrenergic stimulation on vascular SMCs is mediated by HB-EGF transactivation of the EGF receptor (EGFR)[14]. Certain other GPCRs and receptor tyrosine kinases potentially involved in FINR may also signal through HB-EGF[15]. Membrane-anchored pro-HB-EGF is cleaved by the metalloproteinase ADAM17[16] (TACE, TNFα converting enzyme— the major sheddase for HB-EGF)[17] and by matrix metaloproteases (MMPs)[18]. Soluble HB-EGF binds ErbB1 (EGF receptor (EGFR)) and ErbB4[18] and stimulates SMC and fibroblast proliferation and migration with potencies comparable to PDGF-B[18] Pro-HB-EGF is induced by low shear stress in cultured ECs[19] and at aneurysms *in vivo*[20]. Balloon injury also activates HB-EGF in media and neointimal cells[21], and neutralizing antibody to EGFR reduced medial SMC proliferation and intimal hyperplasia[22]. HB-EGF-dependent transactivation of EGFR by certain GPCRs, ie, the AT $_1$-R[15,23] contributes to vascular remodeling, which may result from increased GPCR signaling produced by EC dysfunction[3, 4].

Atherogenesis has been associated with increased HB-EGF activity. Plasma HB-EGF is elevated in coronary artery disease[18]. Proliferation of SMCs induced by remnant lipoproteins is accompanied by HB-EGF shedding and EGFR transactivation[24]. HB-EGF induces expression of oxidized LDL receptor-1[25], is released by activated leukocytes— especially macrophages, and is further elevated by oxidized LDL[25]. Expression of pro-HB-EGF, EGFR and TACE is increased in shoulder regions of atheromas[17,26].

Collectively, these findings suggest HB-EGF may be a key signaling nexus in vascular trophic responses to disturbed shear stress, GPCR signaling, injury and athero-inflammation. Thus, the purpose of the present study was to test the hypothesis that HB-EGF signaling contributes to flow-mediated remodeling.

Materials and Methods

See the online supplement for additional Materials and Methods: http://circres.ahajournals.org/

Wild-type littermates and mice targeted for EGFR ligands (C57Bl/6x129Sv background) are detailed in the online supplement. The left external carotid, internal carotid and occipital arteries were ligated, with the thyroid artery left intact. Carotids were harvested 21 days later. Administration of AG1478-mesylate[27] was via mini-pump and apocynin via drinking water. Carotids were maximally dilated and perfusion-fixed for histology. *In situ* ROS detection was by dihydroethidium or MitoTracker Red. Real-time RT-PCR used 18S for normalization. Leukocyte chemotaxis to 4-O-tetradecanoyphorbol-13-acetate (TPA) was by transwell assay using cells harvested from the peritoneum 4 days after thioglycollate.

Results

Flow-induced negative hypertrophic remodeling (FINR) is dependent on HB-EGF signaling

Twenty-one days after ligation, the left carotid of wild-type mice exhibited a 28% decrease in lumen area, 325% and 142% increases in intima-media and adventitia thickness, respectively, and a 12% increase in circumference of the external elastic laminus (EEL) (Figs 1A and 2A). FINR was partially inhibited in HB-EGF$^{-/+}$ mice (excepting lumen reduction), which express ~50% less HB-EGF[28], and further inhibited in HB-EGF$^{-/-}$ mice (Fig 2A), suggesting that HB-EGF mediates FINR in a dose (allele)-dependent manner. Increased right carotid flow caused eutrophic outward remodeling (Fig 2B, control group). Outward remodeling was unaltered in HB-EGF$^{+/-}$ or HB-EGF$^{-/-}$ mice (Fig 2B), which provides an internal control for specificity.

We also evaluated amphiregulin and betacellulin— other EGF family ligands that bind EGFR. Pro-amphiregulin, like pro-HB-EGF, is proteolytically cleaved by TACE to yield mature growth factor, whereas betacellulin is shed by ADAM10[18]. Neither FINR nor right carotid outward remodeling was altered in amphiregulin$^{-/-}$ or betacellulin$^{-/-}$ mice (Fig 2). These results underscore the specificity of HB-EGF in FINR.

Control for cardiac hypertrophic phenotype in HB-EGF $^{-/-}$ mice

HB-EGF$^{-/-}$ mice are born with cardiac valve deformities, modest reduction in lung alveolar number and increase in pulmonary interstitial tissue[29]. The valvulopathy leads, with varying penetrance, to stenosis, cardiac hypertrophy and progression to heart failure with aging[29]. No effects have been associated with the pulmonary changes[29]. Heart failure could affect FINR through hemodynamic and/or humoral disturbances. However, we did not find any evidence of heart failure: (1) In a separate experiment, electromagnetic flowmetry (Transonic) in isoflurane anesthetized 4.5 month-old mice showed that carotid flows were comparable in wildtype and HB-EGF$^{-/-}$ before and after ligation (Figure 1B), in agreement with their similar arterial pressures (see below). (2) We also correlated heart and lung weight with FINR 21 days after ligation in several strains (Suppl Fig 1A). Only HB-EGF$^{-/-}$ mice had cardiac hypertrophy. However this was without pulmonary edema, ie, lung dry/wet weight in wildtype = 0.086 ±0.0024 and in HB-EGF$^{-/-}$ = 0.079±0.006 (p=0.30; n=5+5), which were comparable to the other groups (Suppl Fig 1A). The small increase in dry weight in HB-EGF$^{+/-}$ and HB-EGF$^{-/-}$ likely reflects the phenotypic increase in pulmonary interstitium[29]. (3) We previously reported[30] similar heart-lung data in HB-EGF$^{-/-}$ mice that were older (5 months), where cardiac hypertrophy was greater (74% versus the 56% in Suppl Fig 1B) and arterial pressure, heart rate and renin-angiotensin activity in conscious unrestrained and anesthetized states were not different from sham-treated wild-type littermates, arguing against heart failure. These and the present results indicate that the HB-EGF$^{-/-}$ mice studied herein had compensatory hypertrophy that had not progressed to congestive heart failure. (4) This conclusion is supported by comparable percentage decreases in body weight before versus 3 weeks after surgery in wild-type (-1.5±2.6%) and HB-EGF$^{-/-}$ (-1.9±1.3) (p=0.99). (5) Moreover, no correlations were present between any parameter of FINR and the amount of cardiac hypertrophy in HB-EGF$^{-/-}$ (Suppl Fig 1B). (6) In addition, outward remodeling in right carotid was not affected

in any group (Fig 2B). (7) Lastly and importantly, FINR was partially inhibited in HB-EGF$^{+/-}$ and AG1478-treated mice (discussed below) despite absence of hypertrophy. Thus, inhibition of FINR by genetic or pharmacologic reduction of HB-EGF signaling (Figs 1, 2A) cannot be ascribed to a secondary effect caused by cardiac hypertrophy.

HB-EGF expression, EFGR and Erk1/2 are augmented during FINR

The above data support the following pathway: low flow → HB-EGF → EGFR → Erk1/2 → hypertrophic remodeling. To examine major elements in this pathway, we first quantified pro-HB-EGF mRNA,as done by others[17,19-21,26]. Pro-HB-EGF increased 2.2 and 4.7 fold at 36 and 72 hours after ligation (Fig 3A). Immunohistochemistry detected increased activity in ECs and diffusely in the media (Fig 3B; see Suppl Fig 2 for positive and negative controls). Since HB-EGF binding to EGFR induces phosphorylation[18], we measured EGFR phosphorylation. Adult male Sprague-Dawley rats were used rather than mice because of availability of antibodies against rat EGFR and protein quantity required for immuno-precipitation/-blot for results shown in Figs 3B-D, Fig 5 and Fig 8A. FINR in rats and mice are similar[9], however the mechanisms may not be identical. Phospho-EGFR increased transiently at 36 hours (Figs 3C,D). In normal carotid artery (sham surgery) immunoreactivity was detected in ECs and medial SMCs close to the lumen, but not adventitial cells which are mostly fibroblasts (Fig 3C). However, phospho-EGFR-positive cells were evident in all layers after ligation, including mono/macrophage-like cells in the adventitia and lumen (Fig 3C). Erk1/2, which is activated by EGFR and important in vascular wall growth and remodeling[14], exhibited sustained activation (Fig 3D).

Inhibition of EGFR reduces FINR

HB-EGF binds EGFR (ErbB1) and ErbB4, and ECs and monocytes/macrophages express EGFR, whereas medial SMCs express all four receptors (ErbB1-B4)[18]. To examine EGFR involvement, AG1478 mesylate, a highly selective inhibitor of EGFR tyrosine kinase[15], was administered via subcutaneous mini-pump. Mesylated AG1478 has increased solubility[27] that was further increased by Captisol[27]. AG1478 inhibited intima-media thickening by 54%, circumference increase by 91%, and adventitial thickening by 58%, but lumen decrease was unaffected (Fig 3E). Partial inhibition of FINR may reflect incomplete blockade of EGFR (see Discussion). We did not test a higher dose because body weight declines[27]; herein it was unaffected. Consistent with results obtained in HB-EGF$^{+/-}$ and HB-EGF$^{-/-}$ mice (Fig 2), right carotid outward remodeling was unaffected by AG1478 (data not shown).

HB-EGF$^{-/-}$ mice display reduced proliferation and leukocyte infiltration

Proliferation and leukocyte accumulation occur early in FINR[9,13]. Likewise, wild-type mice had increased proliferation in intima, media and adventitia, and leukocyte accumulation in lumen, media and adventitia at day-5, while overall cell density did not increase (Fig 4) because proliferation and apoptosis increase similarly[13]. In HB-EGF$^{-/-}$ mice, proliferation in media and leukocyte accumulation in media and adventitia were reduced (Fig 4).

HB-EGF and EGFR are expressed by ECs, SMCs, circulating leukocytes and bone marrow hematopoietic cells[18]. It is not known if HB-EGF expression in the latter cells influences circulating leukocytes. Complete blood counts (CBC) were done to determine if reduced leukocyte accumulation during FINR in HB-EGF$^{-/-}$ mice might arise from diminished plasma levels. No significant differences were found between wild-type and HB-EGF$^{-/-}$ in total WBCs (4.6±1.0 vs 5.1±0.6 $\times 10^3/\mu l$), granulocytes (0.9±0.2 vs 0.9±0.1), lymphocytes (3.0±0.8 vs 3.7 ±0.5) or monocytes (0.7±0.1 vs 0.6±0.1) (n = 5-6). To determine whether leukocytes migration is reduced in HB-EGF$^{-/-}$ mice to explain reduced accumulation, we examined TPA-induced chemotaxis of leukocytes isolated from the peritoneum. No difference was found in transwell migration between wild-type and HB-EGF$^{-/-}$ mice (Fig 4D). These data are consistent with

evidence that increased local tissue HB-EGF promotes macrophage accumulation[31] and suggest that reduced leukocytes in carotids of HB-EGF$^{-/-}$ mice undergoing FINR (Fig 4B) extends from lack of HB-EGF production by vascular wall cells.

Activation of potential upstream effectors of HB-EGF during FINR

The above results suggest that low shear stress activates HB-EGF signaling. To further explore this pathway, we examined TACE— the primary metalloproteinase implicated in HB-EGF shedding[16]. TACE immunoreactivity increased transiently, with a time course that preceded EGFR activation and, like phospho-EGFR (Fig 3D), returned to control by day-5 (Fig 5). TACE immunoreactivity (Fig 5) also followed a pattern similar to EGFR (Fig 3C), ie, staining in the intima and inner layers of the media in sham-ligated carotid, as well as in circulating monocytic cells, increased 36h after ligation. Like HB-EGF and EGFR, little staining was evident in adventitial fibroblasts. The data in figures 4 and 6 suggest leukocytes, ECs and SMCs are involved in TACE → HB-EGF → EGFR signaling in FINR.

No studies have examined whether reduced shear stress enhances ROS *in vivo*, although this has been observed in cultured endothelial cells[32]. We thus examined intracellular ROS by *in situ* staining for dihydroethidium which binds ROS and concentrates it in the nucleus. Ligation increased ROS in the low-flow left carotid and also, as expected (positive control)[33], in the high-flow right carotid (Fig 6A-D). ROS activity in adherent monocytic cells was also evident in the left carotid, along with in ECs (Fig 6C). However, mitochondrial ROS was not increased (Figs 6E,F, see Suppl Fig 3 for quantification). Since NAD(P)H oxidase is the major source of inducible ROS in vascular wall cells, we tested FINR in mice receiving apocynin (an inhibitor of oxidase subunit assembly) in the drinking water. Apocynin inhibited intima-media and adventitia hypertrophy and increase in circumference, but had no effect on lumen reduction (Fig 7A). Essentially identical results were obtained in p47$^{phox-/-}$ mice deficient, a regulatory subunit of the oxidase (Fig 7B). Similar to the HB-EGF$^{-/-}$, HB-EGF$^{+/-}$ and AG1478 experiments, outward remodeling of the right carotid artery was unaffected by apocynin or in p47$^{phox-/-}$ mice (data not shown).

Evidence in ECs and SMCs suggests that NF-κB is activated by low/disturbed shear stress[32], ROS[34], IL-8 and TNF-α[18] and that these factors increase pro-HB-EGF expression in part through NF-κB. We thus examined whether NF-κB is activated (p65 subunit translocation) during reduced flow. In ECs of carotid arteries with normal flow, p65 immunoreactivity appeared abundant in the cytoplasm but absent in the nucleus (Suppl Fig 4). However, 6h after ligation, nuclear translocation appeared evident. Staining was weak in medial SMCs and adventitial fibroblasts in both normal and low-flow conditions, precluding detection of translocation (Suppl Fig 4). Since this assay is limited for quantification, NF-κB activation in both ECs and SMCs was confirmed with semi-quantitative immunohistochemistry for activated p65 (Fig 8A,B). To determine whether HB-EGF is upstream or downstream of NF-κB, we also compared activated NF-κB in wild-type and HB-EGF$^{-/-}$ mice 5 days after ligation (Fig 8B,C). NF-κB activation in ECs of wild-type and HB-EGF$^{-/-}$ were similar; however, activation was reduced in SMCs of HB-EGF$^{-/-}$ mice. These data demonstrate that a more rapid and sustained activation of NF-κB activation occurs in ECs compared to SMCs during low flow, and suggest that HB-EGF may reside, relative to NF-κB, downstream in ECs and upstream in SMCs.

Discussion

Evidence suggests flow-induced arterial remodeling involves factors released from cells that are intrinsic to the vessel wall and recruited from the bloodstream. Understanding the molecular details has been hampered by the need to study the process *in vivo*. The present findings suggest that HB-EGF, which has primarily been studied in epithelial and tumor cells, plays a pivotal

role in low flow-induced negative hypertrophic remodeling (FINR) of the mouse carotid artery. Sustained low flow activated or increased the following elements within the HB-EGF signaling pathway: ROS, the ROS-sensitive HB-EGF sheddase TACE, expression of pro-HB-EGF, HB-EGF immunoreactivity, the HB-EGF receptor EGFR, Erk1/2, and the transcription factor NF-κB. These changes were associated with proliferation, increased leukocyte density, wall hypertrophy and lumen narrowing. Heterozygous and homozygous deletion of HB-EGF alleles caused "dose-dependent-like" inhibition of FINR (although inhibition of lumen narrowing was in some situations spared (see below), where proliferation and leukocyte accumulation were reduced and FINR almost abolished in HB-EGF$^{-/-}$. Inhibition was also obtained by genetic and pharmacologic reduction in ROS generation and inhibition of EGFR activation. FINR was unaffected in mice deficient in the EGFR ligand family members, betacellulin and amphiregulin.

Interestingly, none of the above interventions affected high flow-induced eutrophic positive remodeling of the right carotid. This suggests that low and high flow-mediated remodeling are achieved by unique mechanisms. A second intriguing observation is that in FINR, significant inhibition of wall hypertrophy in HB-EGF$^{+/-}$ and p47$^{phox-/-}$ mice, and with AG1478 or apocynin treatment, did not inhibit lumen narrowing (an exception was in HB-EGF$^{-/-}$ mice, but their baseline diameters trended lower for unapparent reasons). This suggests that HB-EGF signaling is necessary for the hypertrophic response in FINR, but not for the mechanisms that cause lumen narrowing. The latter mechanisms may be driven in a negative feedback manner to normalize shear stress. This is the first report identifying a role for the HB-EGF pathway in flow-induced remodeling.

Although HB-EGF is well-known to activate EGFR and downstream mechanisms including Akt and Erk1/2 in SMCs, ECs and other cell types, few studies have investigated possible links between reduced shear stress, ROS and HB-EGF activation. Reduced shear stress causes rapid increase in HB-EGF expression in ECs[19]. Low or disturbed shear stress promotes inflammatory-like conditions associated with increased ROS[32], in particular reduced nitric oxide[35], increased expression of attractant and adhesion molecules that promote leukocyte transmigration[3], and increased local angiotensin, endothelin and norepinephrine activity that induce ROS- and HB-EGF-dependent GPCR transactivation of EGFR[14,15,23]. Although angiotensin and endothelin have not been examined in FINR, norepinephrine-induced trophic activity contributes significantly to FINR[13]. In agreement with the prominent role of SMC proliferation in FINR[7,10,15], proliferation was inhibited in the media but not intima in HB-EGF$^{-/-}$ mice (Fig 4A)— a finding consistent with HB-EGF's mitogenic action on SMCs but not ECs[18]. Besides SMCs, leukocytes and ECs (and possibly adventitial fibroblasts) also release HB-EGF and express EGFR *in vitro*[18,19]. Indeed, our histological results confirm ROS, TACE and EGFR activity in each vascular layer. Thus, autacrine/paracrine HB-EGF activity in FINR could involve all four cell types. Additional studies are required to determine their relative contributions. It should be noted that only associative evidence is provided for two of the elements (TACE, Erk1/2) in the signaling pathway proposed herein, because in the case of TACE specific antagonists and genetic models (TACE$^{-/-}$ mice are embryonic lethal) are currently unavailable.

Primarily *in vitro* studies have shown that pro-HB-EGF is cleaved by ADAM family proteases and MMPs[18]. Evidence suggests that TACE (ADAM-17) is the primary mediator of HB-EGF shedding in many cells types, including SMCs where proliferation and hypertrophy result[23]. In the present study, TACE immunoreactivity was elevated at the earliest time point examined after flow reduction (12 hr). ROS can increase MMP expression through Erk1/2, JNK, AP-1 and Ets-1[36]. While this pathway has not been examined for TACE, TACE may be directly activated by a ROS-sensitive cysteine switch mechanism[37]. In the present study, systemic administration of the NAD(P)H oxidase inhibitor apocynin, as well as p47$^{phox-/-}$ mice, resulted

in potent inhibition of FINR, while increased mitochondrial ROS activity was not evident. Although the role of other sources of ROS and the specific ROS molecule(s) and pro-HB-EGF protease(s) that direct FINR remain to be determined, our findings demonstrate a primary role for NAD(P)H oxidase.

The observation that pro-HB-EGF expression occurs rapidly (hours) in response to reduced shear stress in ECs[19] and to other stimuli in other cells, suggests that HB-EGF behaves as an immediate-early gene[21]. The HB-EGF promoter contains a consensus site for NF-κB[21]. Binding of NF-κB is redox sensitive, and activation of NF-κB by TNF-α and IL-β has been linked to induction of ROS molecules[34]. Indeed, low shear stress in cultured ECs activates NF-κB by a NAD(P)H oxidase-dependent mechanism[32]. Our results support the presence of this signaling sequence in arteries exposed to low flow *in vivo*. Interestingly, in HB-EGF$^{-/-}$ mice, activation of NF-κB was unaffected in ECs but abolished in SMCs (Fig 8D), suggesting that low shear-induced activation of NF-κB is upstream of HB-EGF in ECs but downstream in SMCs. Consistent with this, in SMCs NF-κB did not induce expression of EGFR mRNA or protein[38], whereas EGFR stimulation induced NF-κB activation[39]. Others have found that NF-κB in SMCs resides downstream of EGFR in a pathway inducing Erk1/2 and Akt mediated proliferation and apoptosis[40]. Also, peak activation of NF-κB occurred earlier in ECs than in SMCs (Fig 8B), possibly because ECs are more directly coupled mechanically to fluid shear stress than are SMCs.

Besides ECs and SMCs, FINR-induced leukocyte accumulation is also a potential source of HB-EGF[18,26]. Activated macrophages exhibit increased HB-EGF expression and release[18, 26]. In the present study we confirmed earlier reports[13] for leukocyte accumulation in intima, media and adventitia during FINR, and detected reduction in HB-EGF$^{-/-}$ mice. Interestingly, HB-EGF promotes macrophage accumulation in ischemic heart[31]. Thus, HB-EGF release from vascular wall cells may contribute to recruitment of inflammatory cells that further increase local HB-EGF activity. We did not find that reduced leukocyte density in HB-EGF$^{-/-}$ mice was accompanied by reduced circulating leukocyte counts or migratory capacity. However, pharmacological depletion of macrophages reduced FINR of small branches of the mesenteric artery[12]. Whether the association of reduced leukocyte density and reduced FINR in that study[12] and the present study extends from less HB-EGF in the wall coming from fewer infiltrated cells— or from the attendant lower levels of cytokines such as IL1/6 and TNFα that induce HB-EGF in vascular wall cells— awaits studies using selective leukocyte depletion of HB-EGF.

Partial inhibition of FINR was obtained with chronic systemic administration of the EGFR antagonist AG1478-mesylate (Fig 3E). We did use a higher dose to test for incomplete blockade because of potential non-specific effects[27]. However, ErbB receptor-ligand interactions could also underlie the partial inhibition. ErbB1 (EGFR) and ErbB4 have different biological functions[18]. Proliferation is mediated by ErbB1, whereas chemotaxis is mediated by ErbB4[18]. Also, ErbB2 (for which no ligand has been identified) forms heterodimers with ErbB1 or ErbB4 which may induce cross-talk among downstream pathways promoting proliferation or migration[18]. Neither ErbB2 nor ErbB4 are blocked by AG1478. In addition, Higashiyama and coworkers have recently identified an intracellular signaling pathway activated by pro-HB-EGF cleavage[41], wherein the intracellular carboxy-terminal remnant (HB-EGF-C) interacts with the transcriptional regulator, promyelocytic leukemia zinc finger (PLZF), resulting in increased proliferation. This mechanism, which is independent of EGFR— thus not inhibited by AG1478— could contribute to the residual FINR in the presence of AG1478 that we observed.

In conclusion, the present study has identified a central role for ROS→ HB-EGF→ EGFR signaling in hypertrophic low flow-induced arterial remodeling. Besides physiological

remodeling, this pathway may also contribute to pathological processes. For example, oxidized LDL and remnant lipoproteins induce HB-EGF[24], and HB-EGF increases expression of oxidized LDL receptor[25]. TACE, pro-HB-EGF and EGFR are increased in human atheromas and in those of animals with experimentally induced atherosclerosis[17,26]. And plasma HB-EGF is increased in patients with atherosclerosis[18]. It is therefore intriguing to hypothesize that increased HB-EGF signaling might impair the important adaptive outward remodeling response that occurs at sites of expanding atheromas along arteries[3]. If this were true, inhibition of pathway activity might enhance this process and promote preservation of lumen area. Our finding that HB-EGF signaling is not involved in flow-induced positive remodeling suggests that blocking adverse effects of excessive HB-EGF activity may leave this important physiological mechanism intact.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Carolyn Suitt for histological assistance, Wendy Salmon for assistance with confocal microscopy, and Drs. Nageswara Madamanchi and Marshall Runge, UNC, for $p47^{phox-/-}$ mice.

Sources of Funding This work was supported by grants HL62584 to JEF and CA43793 to DCL and SWS.

REFERENCES

1. Ward MR, Pasterkamp G, Yeung AC, Borst C. Arterial remodeling. Mechanisms and clinical implications. Circulation 2000;102:1186–1191. [PubMed: 10973850]
2. Prior BM, Yang HT, Terjung RL. What makes vessels grow with exercise training? J Appl Physiol 2004;97:1119–1128. [PubMed: 15333630]
3. Cunningham KS, Gotlieb AI. The role of shear stress in the pathogenesis of atherosclerosis. Lab Invest 2005;85:9–23. [PubMed: 15568038]
4. Chatzizisis Y, Giannoglou G. Pulsatile flow: a critical modulator of the natural history of atherosclerosis. Med Hypotheses 2006;67:338–340. [PubMed: 16546326]
5. Stone P, Coskun A, Yeghiazarians Y, Kinlay S, Popma J, Kuntz R, Feldman C. Prediction of sites of coronary atherosclerosis progression: In vivo profiling of endothelial shear stress, lumen, and outer vessel wall characteristics to predict vascular behavior. Curr Opin Cardiol 2003;18:458–470. [PubMed: 14597887]
6. Silver A, Vita J. Shear-stress-mediated arterial remodeling in atherosclerosis: too much of a good thing? Circulation 2006;113:2787–2789. [PubMed: 16785350]
7. Korshunov VA, Mohan AM, Georger MA, Berk BC. Axl, a receptor tyrosine kinase, mediates flow-induced vascular remodeling. Circ Res 2006;98:1446–1452. [PubMed: 16627783]
8. Fortuno A, Jose G, Moreno M, Diez J, Zalba G. Oxidative stress and vascular remodelling. Exp Physiol 2005;90:457–462. [PubMed: 15890797]
9. Sullivan C, Hoying J. Flow-dependent remodeling in the carotid artery of fibroblast growth factor-2 knockout mice. Arterioscler Thromb Vasc Biol 2002;22:1100–1105. [PubMed: 12117723]
10. Korshunov VA, Berk BC. Strain-dependent vascular remodeling: the “Glagov phenomenon” is genetically determined. Circulation 2004;110:220–226. [PubMed: 15226209]
11. Yu J, Bergaya S, Murata T, Alp I, Bauer M, Lin M, Drab M, Kurzchalia T, Stan R, Sessa W. Direct evidence for the role of caveolin-1 and caveolae in mechanotransduction and remodeling of blood vessels. J Clin Invest 2006;116:1284–1291. [PubMed: 16670769]
12. Bakker E, Pistea A, Spaan J, Rolf T, de Vries C, van Rooijen N, Candi E, VanBavel E. Flow-dependent remodeling of small arteries in mice deficient for tissue-type transglutaminase: possible compensation by macrophage-derived factor XIII. Circ Res 2006;99:86–92. [PubMed: 16741156]

13. Erami C, Zhang H, Tanoue A, Tsujimoto G, Thomas S, Faber J. Adrenergic catecholamine trophic activity contributes to flow-mediated arterial remodeling. Am J Physiol Heart Circ Physiol 2005;289:H744–753. [PubMed: 15849236]

14. Zhang H, Chalothorn D, Jackson L, Lee D, Faber J. Transactivation of epidermal growth factor receptor mediates catecholamine-induced growth of vascular smooth muscle. Circ Res 2004;95:989–997. [PubMed: 15486316]

15. Touyz R. Mitochondrial redox control of matrix metalloproteinase signaling in resistance arteries. Arterioscler Thromb Vasc Biol 2006;26:685–688. [PubMed: 16556862]

16. Sunnarborg S, Hinkle C, Stevenson M, Russell W, Raska C, Peschon J, Castner B, Gerhart M, Paxton R, Black R, Lee D. Tumor necrosis factor-alpha converting enzyme (TACE) regulates epidermal growth factor receptor ligand availability. J Biol Chem 2002;277:15.

17. Canault M, Peiretti F, Kopp F, Bonardo B, Bonzi M, Coudeyre J, Alessi M, Juhan-Vague I, Nalbone G. The TNF alpha converting enzyme (TACE/ADAM17) is expressed in the atherosclerotic lesions of apolipoprotein E-deficient mice: possible contribution to elevated plasma levels of soluble TNF alpha receptors. Atherosclerosis 2006;187:82–91. [PubMed: 16214147]

18. Dreux A, Lamb D, Modjtahedi H, Ferns G. The epidermal growth factor receptors and their family of ligands: their putative role in atherogenesis. Atherosclerosis 2006;186:38–53. [PubMed: 16076471]

19. Morita T, Yoshizumi M, Kurihara H, Maemura K, Nagai R, Yazaki Y. Shear stress increases heparin-binding epidermal growth factor-like growth factor mRNA levels in human vascular endothelial cells. Biochem Biophys Res Commun 1993;197:256–262. [PubMed: 8250932]

20. Sho E, Sho M, Nanjo H, Kawamura K, Masuda H, Dalman R. Comparison of cell-type-specific vs transmural aortic gene expression in experimental aneurysms. J Vasc Surg 2005;41:844–852. [PubMed: 15886670]

21. Igura T, Kawata S, Miyagawa J, Inui Y, Tamura S, Fukuda K, Isozaki K, Yamamori K, Taniguchi N, Higashiyama S, Matsuzawa Y. Expression of heparin-binding epidermal growth factor-like growth factor in neointimal cells induced by balloon injury in rat carotid arteries. Arterioscler Thromb Vasc Biol 1996;16:1524–1531. [PubMed: 8977458]

22. Chan A, Kalmes A, Hawkins S, Daum G, Clowes A. Blockade of the epidermal growth factor receptor decreases intimal hyperplasia in balloon-injured rat carotid artery. J Vasc Surg 2003;37:644–649. [PubMed: 12618705]

23. Ohtsu H, Dempsey P, Frank G, Brailoiu E, Higuchi S, Suzuki H, Nakashima H, Eguchi K, Eguchi S. ADAM17 mediates epidermal growth factor receptor transactivation and vascular smooth muscle cell hypertrophy induced by angiotensin II. Arterioscler Thromb Vasc Biol 2006;26:e133–137. [PubMed: 16840716]

24. Kawakami A, Yoshida M. Remnant lipoproteins and atherogenesis. J Atheroscler Thromb 2005;12:73–76. [PubMed: 15942116]

25. Mukai E, Kume N, Hayashida K, Minami M, Yamada Y, Seino Y, Kita T. Heparin-binding EGF-like growth factor induces expression of lectin-like oxidized LDL receptor-1 in vascular smooth muscle cells. Atherosclerosis 2004;176:289–296. [PubMed: 15380451]

26. Nakata A, Miyagawa J, Yamashita S, Nishida M, Tamura R, Yamamori K, Nakamura T, Nozaki S, Kameda-Takemura K, Kawata S, Taniguchi N, Higashiyama S, Matsuzawa Y. Localization of heparin-binding epidermal growth factor-like growth factor in human coronary arteries. Possible roles of HB-EGF in the formation of coronary atherosclerosis. Circulation 1996;94:2778–2786. [PubMed: 8941102]

27. Johns T, Luwor R, Murone C, Walker F, Weinstock J, Vitali A, Perera R, Jungbluth A, Stockert E, Old L, Nice E, Burgess A, Scott A. Antitumor efficacy of cytotoxic drugs and the monoclonal antibody 806 is enhanced by the EGF receptor inhibitor AG1478. Proc Natl Acad Sci U S A 2003;100:15871–15876. [PubMed: 14676326]

28. Iwamoto R, Yamazaki S, Asakura M, Takashima S, Hasuwa H, Miyado K, Adachi S, Kitakaze M, Hashimoto K, Raab G, Nanba D, Higashiyama S, Hori M, Klagsbrun M, Mekada E. Heparin-binding EGF-like growth factor and ErbB signaling is essential for heart function. Proc Natl Acad Sci U S A 2003;100:3221–3226. [PubMed: 12621152]

29. Jackson L, Qiu T, Sunnarborg S, Chang A, Zhang C, Patterson C, Lee D. Defective valvulogenesis in HB-EGF and TACE-null mice is associated with aberrant BMP signaling. EMBO J 2003;22:2704–2716. [PubMed: 12773386]
30. Chalothorn D, Moore S, Zhang H, Sunnarborg S, Lee D, Faber J. Heparin-binding epidermal growth factor-like growth factor, collateral vessel development, and angiogenesis in skeletal muscle ischemia. Arterioscler Thromb Vasc Biol 2005;25:1884–1890. [PubMed: 15994441]
31. Mahtouk K, Jourdan M, De Vos J, Hertogh C, Fiol G, Jourdan E, Rossi J, Klein B. Local overexpression of HB-EGF exacerbates remodeling following myocardial infarction by activating noncardiomyocytes. Lab Invest 2005;85:862–873. [PubMed: 15856048]
32. Mohan S, Koyoma K, Thangasamy A, Nakano H, Glickman R, Mohan N. Low shear stress preferentially enhances IKK activity through selective sources of ROS for persistent activation of NF-kappaB in endothelial cells. Am J Physiol Cell Physiol 2007;292:C362–371. [PubMed: 16914532]
33. Castier Y, Brandes R, Leseche G, Tedgui A, Lehoux S. p47phox-dependent NADPH oxidase regulates flow-induced vascular remodeling. Circ Res 2005;97:533–540. [PubMed: 16109921]
34. Gloire G, Legrand-Poels S, Piette J. NF-kappaB activation by reactive oxygen species: fifteen years later. Biochem Pharmacol 2006;72:1493–1505. [PubMed: 16723122]
35. Mohan S, Hamuro M, Sorescu G, Koyoma K, Sprague E, Jo H, Valente A, Prihoda T, Natarajan M. IkappaBalpha-dependent regulation of low-shear flow-induced NF-kappa B activity: role of nitric oxide. Am J Physiol Cell Physiol 2003;284:C1039–1047. [PubMed: 12620896]
36. Nelson K, Melendez J. Mitochondrial redox control of matrix metalloproteinases. Free Radic Biol Med 2004;37:768–784. [PubMed: 15304253]
37. Zhang Z, Oliver P, Lancaster JJ, Schwarzenberger P, Joshi M, Cork J, Kolls J. Reactive oxygen species mediate tumor necrosis factor alpha-converting, enzyme-dependent ectodomain shedding induced by phorbol myristate acetate. FASEB J 2001;15:303–305. [PubMed: 11156944]
38. Nishi H, Neta G, Nishi KH, Akers LM, Rikiyama T, Proctor KN, Murphy BA, Johnson AC. Analysis of the epidermal growth factor receptor promoter: the effect of nuclear factor-kappaB. Int J Mol Med 2003;11:49–55. [PubMed: 12469217]
39. Sethi G, Ahn KS, Chaturvedi MM, Aggarwal BB. Epidermal growth factor (EGF) activates nuclear factor-kappaB through IkappaBalpha kinase-independent but EGF receptor-kinase dependent tyrosine 42 phosphorylation of IkappaBalpha. Oncogene 2007;26:7324–32. [PubMed: 17533369]
40. Lemarie C, Tharaux P, Esposito B, Tedgui A, Lehoux S. Transforming growth factor-alpha mediates nuclear factor kappaB activation in strained arteries. Circ Res 2006;99:434–441. [PubMed: 16857964]
41. Nanba D, Inoue H, Shigemi Y, Shirakata Y, Hashimoto K, Higashiyama S. An intermediary role of proHB-EGF shedding in growth factor-induced c-Myc gene expression. J Cell Physiol 2008;214:465–73. [PubMed: 17674363]

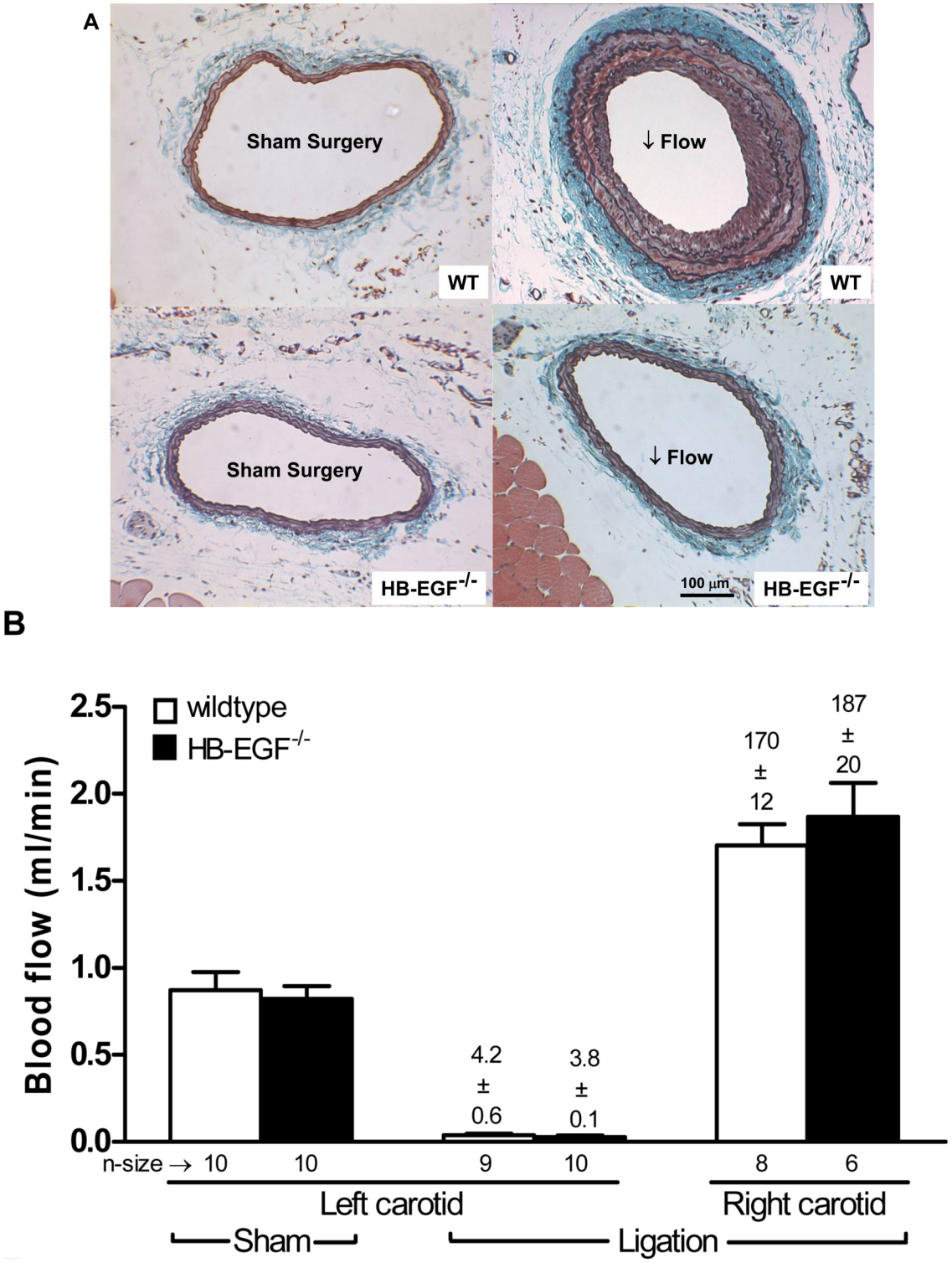


Fig 1.

Low flow-induced negative hypertrophic remodeling (FINR) of left carotid is reduced in HB-EGF$^{-/-}$ mice. **A**. Sections of left common carotids from HB-EGF$^{-/-}$ and wild-type littermates 21 days after reduction of flow; magnification scale bars here and in other figures are the same for all panels in the figure unless indicated otherwise. **B**. Carotid flows (Transonic Inc) were

comparable in wildtype and HB-EGF$^{-/-}$ before (sham) and after ligation. Values for percent of control (sham) are given above columns. Data in this and subsequent figures are given as mean ±SEM for n number of vessels/animals.

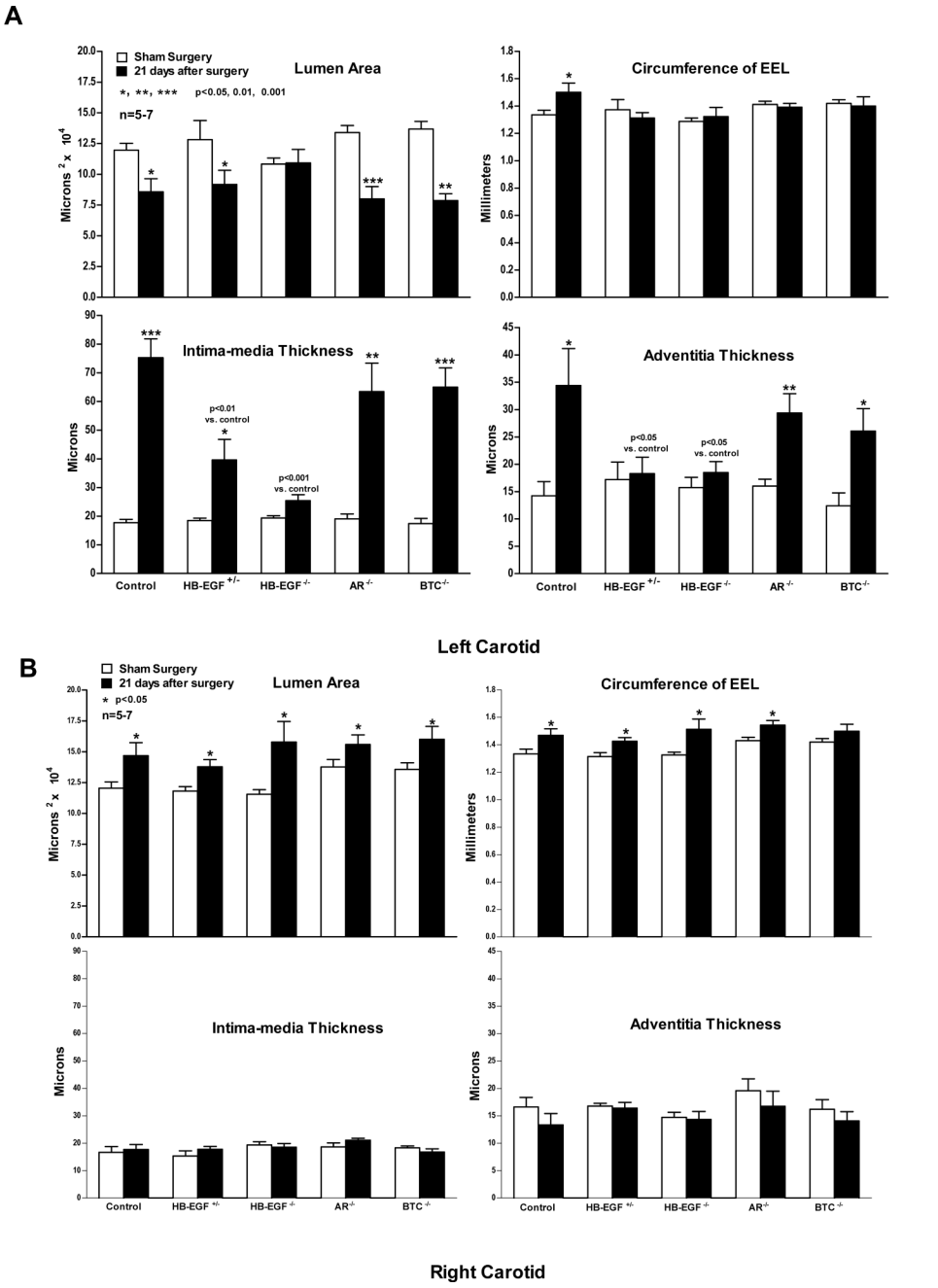


Fig 2.

FINR is reduced in HB-EGF$^{-/-}$ mice. Morphometry was performed 21 days after surgery. **A**. FINR in left carotid was inhibited in HB-EGF$^{+/-}$ and HB-EGF$^{-/-}$, but not in amphiregulin$^{-/-}$ (AR) and betacellulin$^{-/-}$ (BTC) mice. **B.** High flow-induced outward remodeling of right carotid was similar in the genetically deficient groups. Data in this and subsequent figures were subjected to t-tests unless indicated otherwise.

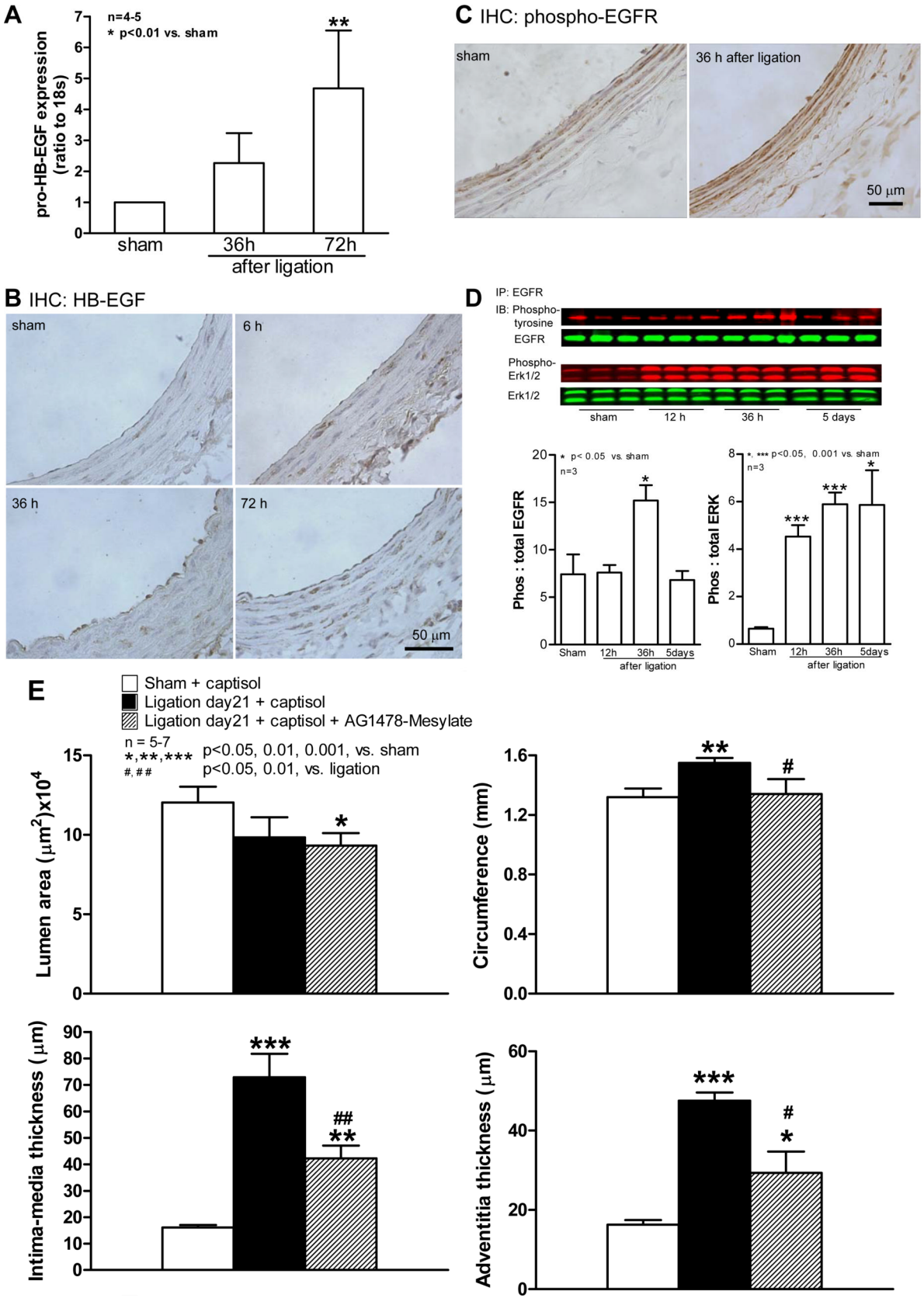


Fig 3.

HB-EGF, EGFR and Erk1/2 are induction during FINR of left carotid arteries. **A.** Pro-HB-EGF mRNA expression in mouse carotid. **B,C.** Immunohistochemistry on cryosections showing increased HB-EGF- and phospho-EGFR-like immunoreactivity in ECs and diffusely across the rat carotid wall after ligation (see Suppl Fig 2 for controls). In sham-treated animals, phospho-EGFR is evident in endothelium and inner media. After ligation, phospho-EGFR staining in all layers. Data are representative of 4-5 sham and 4-5 ligated vessels. **D.** Immunoprecipitation/immunoblot of rat carotid for phospho-EGFR and immunoblot of Erk1/2 activation. Bar graphs give average fluorescent intensity of lanes. **E.** Inhibition of EGFR with AG1478 reduces FINR. AG1478 or vehicle (captisol) was administered to C57BL/6xSv129 mice for 21 days after ligation. AG1478 had no effect on positive remodeling of right carotid (data not shown).

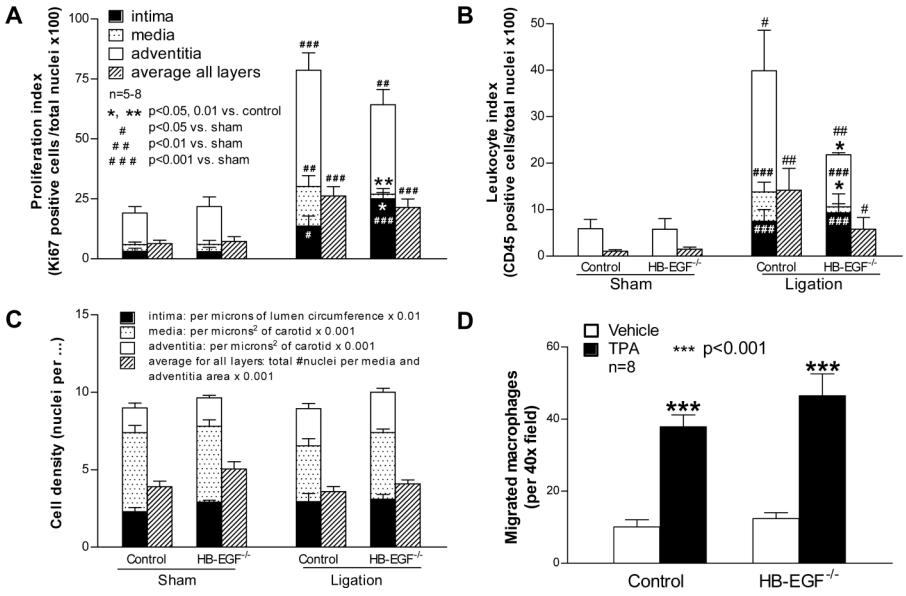


Fig 4.

FINR is associated with proliferation and leukocyte accumulation. Proliferation (Ki67 immunohistochemistry, **A**), leukocyte accumulation (CD45 immunohistochemistry, **B**) and total cell density (hematoxylin and eosin, **C**) for lumen surface (intima), media, adventitia and the average of all 3 layers, 5 days after ligation in mice. Ligation of wild-type mice increased proliferation and leukocyte accumulation in all layers (cell density did not change because of concomitant apoptosis - see Results). Ligation of HB-EGF$^{-/-}$ evidenced less proliferation in media, and less leukocyte accumulation in media and adventitia. **D**, TPA induced *in vitro* chemotaxis of leukocytes is not reduced in HB-EGF$^{-/-}$ mice.

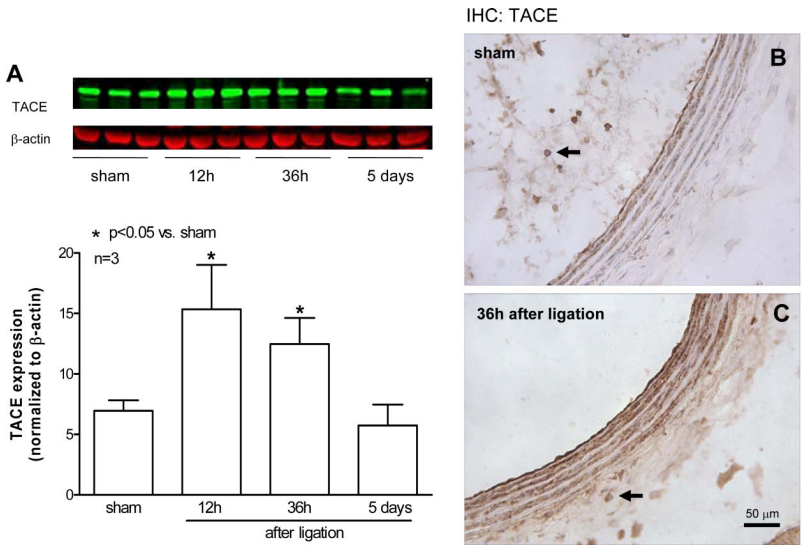


Fig 5.

TACE is increased during FINR of carotid artery. **A.** Immunoblot of TACE in rat left carotids. 10 ug/lane protein loaded onto 10% PAGE gel. **B,C.** Cryosections from rat left carotid arteries demonstrating increased TACE staining. In sham-treated animals, TACE is present in endothelium, inner medial layers and monocytic cells in lumen (arrow). After ligation, increased TACE is evident in intima, media and mono/macrophage-like cells in the adventitia (arrow). This pattern was similar to phospho-EGFR (Fig 3C). Data are representative of 4-5 sham and 4-5 ligated vessels.

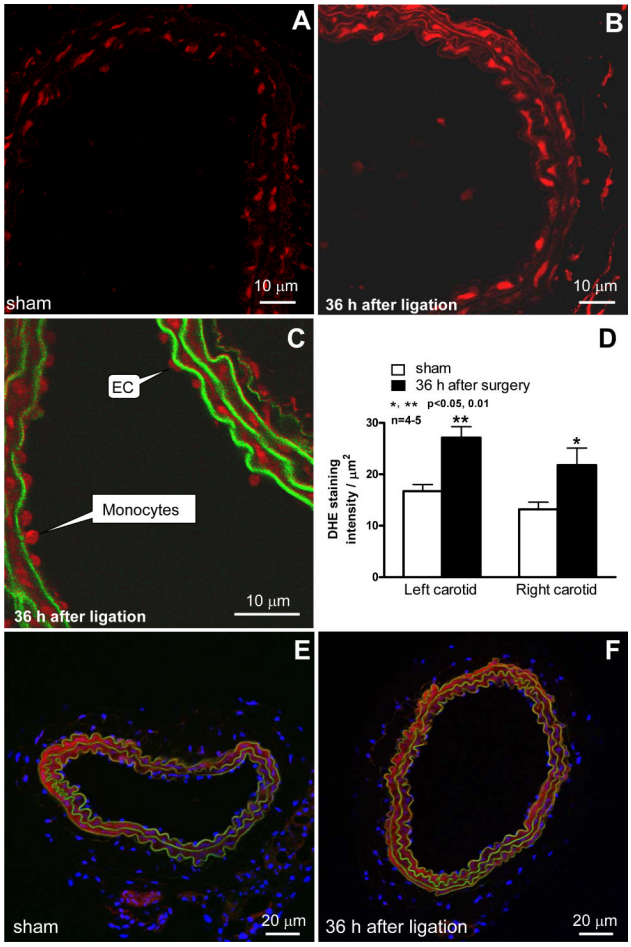


Fig 6.

Fig 6. Reactive oxygen species (ROS) increased in left and right carotid after ligation. ROS detection by confocal dihydroethidium (DHE) staining of mouse left carotid. **A,B.** Increased ROS was detected 36 h after ligation. **C.** Different animal from panel B showing ROS and elastin autofluorescence (green) 36 h after ligation to demarcate intima for identification of ROS activity in EC and monocytes. **D.** Quantification of ROS in carotid arteries. **E,F.** Mitochondrial ROS staining shows no difference between low and normal flow carotids (see Suppl Fig 3 for quantification). Green, elastin; blue, nuclear DAPI staining. Mitochondrial ROS was also not increased in right carotid. Data are representative of 4-5 sham and 4-5 ligated vessels.

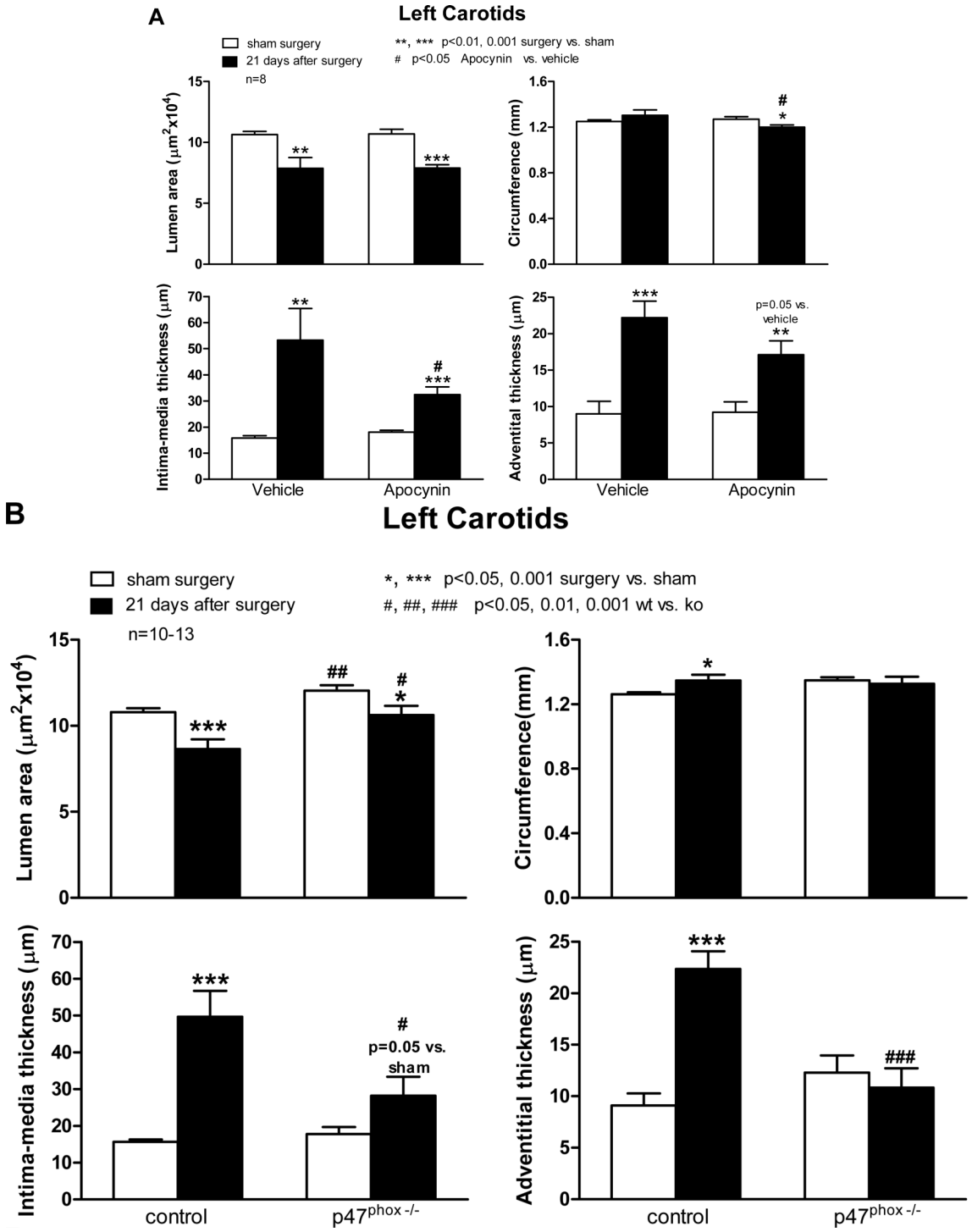
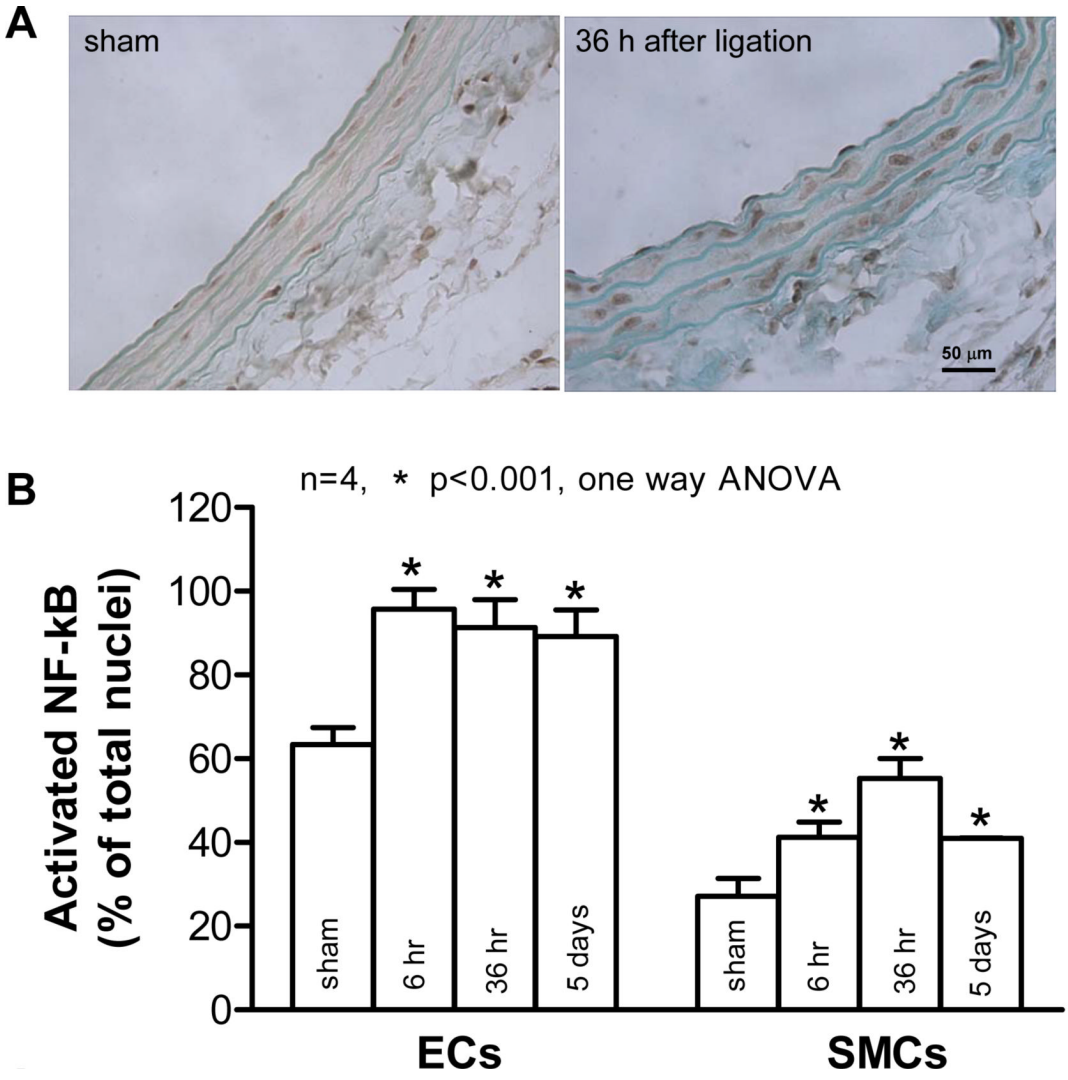


Fig 7.

ROS contributes to low flow-induced remodeling. FINR was reduced by chronic apocynin (**A**) and in p47$^{phox-/-}$ mice (**B**). High flow-induced positive remodeling was not affected in p47$^{phox-/-}$ mice (data not show).



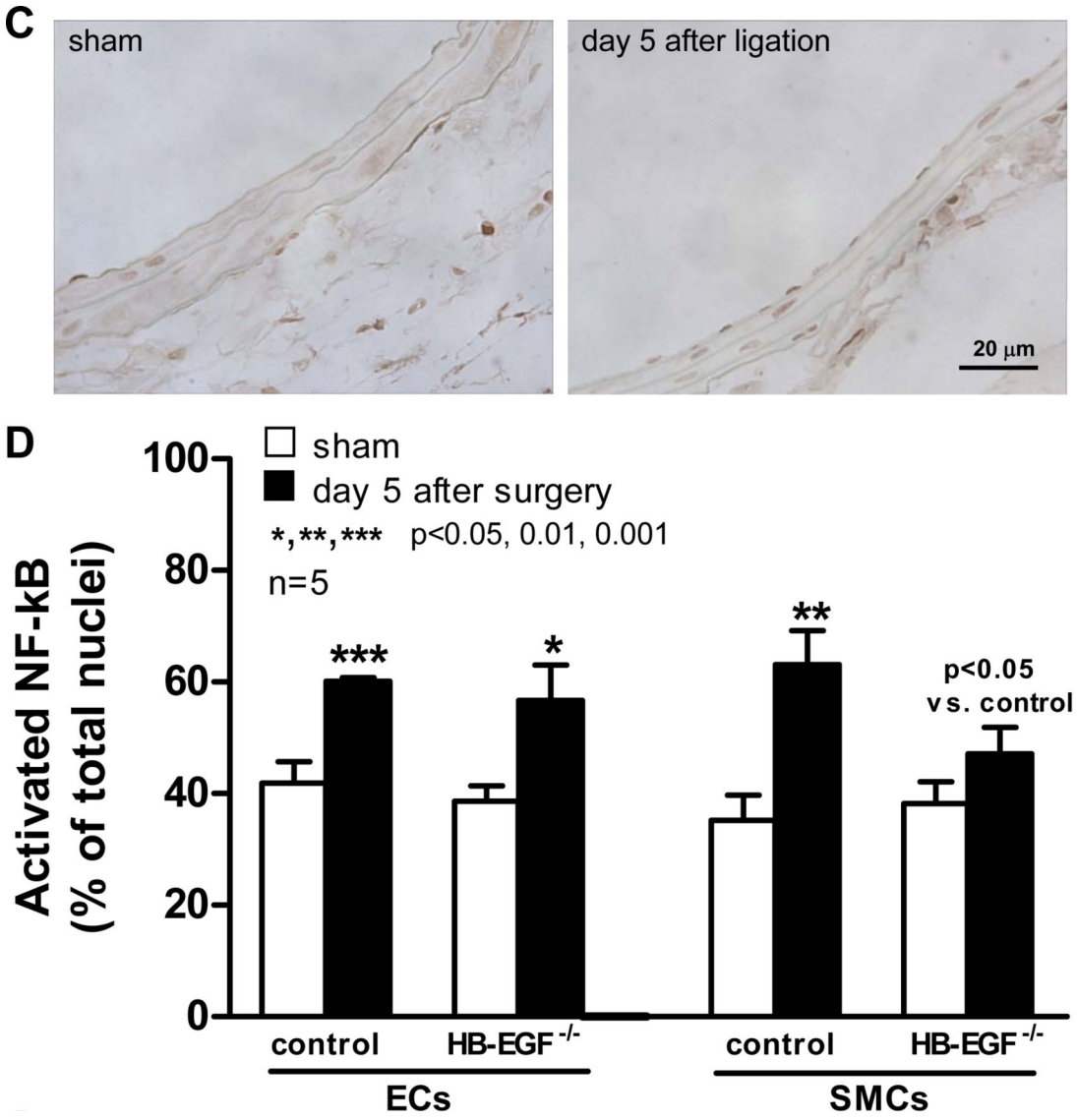


Fig 8.

NF-κB activation during FINR. **A**. Activated NF-κB in rat carotids detected by nuclear-specific NF-κB p65 subunit antibody, showing increased staining after ligation (right section). Quantification given in bar graph (**B**). **C**. Activated NF-κB staining in wild-type mice 5 days after ligation or sham ligation, **D**. Quantification of wild-type (control) and HB-EGF$^{-/-}$ mice. Data for NF-κB translocation assay given in Supplemental figure 4.